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INHIBITION OF GERMINAL VESICLE BREAKDOWN AND ACTIVATION OF CYTOPLASMIC CONTRACTILITY IN *SPISULA* OOCYTES BY CALMODULIN ANTAGONISTS

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Abstract

We have treated *Spisula* oocytes with the calmodulin antagonists, chlorpromazine, calmidazolium, and W-7, and the inactive W-7 analog, W-5, to examine the role of calmodulin in meiosis reinitiation and egg activation in this species. Chlorpromazine and W-7 both inhibited germinal vesicle breakdown (GVBD) at 50–75 μM, whether triggered parthenogenetically by KCl activation or by fertilization. Calmidazolium was effective at *ca.* 3 μM. W-5 was ineffective. However, none of the anticalmodulin drugs inhibited GVBD when induced by ionophore A23187. Time course studies showed that W-7 was effective at inhibiting KCl-induced GVBD only when added within 1 min after KCl. Similar studies using the calcium channel blocker, verapamil, showed that the period during which calcium channels are required for GVBD substantially exceeds the interval of sensitivity to W-7. Calmodulin thus acts at a stage prior to the calcium flux known to be required for GVBD.

Chlorpromazine, calmidazolium, and W-7 triggered ameboid contractions of the immature oocyte in addition to inhibiting GVBD. W-5 was again ineffective. The ameboid contractions observed are not typical of parthenogenetically activated oocytes of this species. Experiments in which chlorpromazine-treated oocytes were simultaneously treated with low concentrations of cytochalasin B showed that the cytoplasmic contractions are microfilament-dependent. The results indicate that the contractions triggered by the calmodulin antagonists are not a part of the normal egg activation process and that cytoplasmic contractions can be induced in this species independently of egg activation and may result from perturbation of a calmodulin-microfilament interaction.

Introduction

Calmodulin has been implicated in the regulation of germinal vesicle breakdown (GVBD) in several species, including starfish (Meijer and Guerrier, 1981; Dorée *et al.,* 1982), frogs (Wasserman and Smith, 1981), annelids (Carroll and Eckberg, 1983), and mice (Bornslaeger *et al.,* 1984). The involvement of calmodulin in the regulation of GVBD may be general and, therefore, basic to a fundamental intracellular mechanism regulating the process of GVBD, because the apparent "triggering" event for GVBD is not the same for all these forms. Starfish and vertebrates undergo GVBD in response to hormonal stimulation, whereas the annelid, *Chaetopterus,* undergoes GVBD upon contact of the oocytes with sea water.

Additionally, calmodulin has been implicated in the control of egg activation in sea urchins (Baker and Whitaker, 1980; Steinhardt and Alderton, 1982) and annelids

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(Carroll and [illegible], 1983). In sea urchins, the criterion for egg activation has been cortical vesicle [illegible]tosis, whereas in the annelid, *Chaetopterus,* the criterion for activation [illegible] set of ameboid contractions characteristic of the early phases of differentiation [illegible]thout cleavage, a usual result of parthenogenetic activation in this species (Lillie, 1902; Eckberg, 1981). In our initial study, treatment with calmodulin antagonists resulted in ameboid contractions more violent than usually observed during differentiation without cleavage.

The present investigation further examined the possible involvement of calmodulin in the regulation of germinal vesicle breakdown by analysis of an organism in which this process is normally triggered by a third mechanism, fertilization, and compared any calmodulin involvement in fertilization-induced GVBD with parthenogenetically initiated GVBD in the same species. The results indicate that calmodulin is involved in GVBD in this species, as well. Analysis of the kinetics of appearance of the inhibitory effect indicates that calmodulin participates in early events of maturation and activation in this species. Studies involving ionophore activation and the calcium channel blocker, verapamil, show that a calcium flux and a calcium-dependent step that is independent of calmodulin follows the calmodulin-dependent period. Additionally, antagonist-induced ameboid contractions were evidently independent of normal egg activation and resulted directly or indirectly from an effect on a microfilament-dependent cytoplasmic contractile system.

Materials and Methods

Mature specimens of *Spisula solidissima* were obtained from the Marine Resources Division, Marine Biological Laboratory, and handled by standard methods (Costello and Henley, 1971). Oocytes were washed 3–4 times with seawater prior to use. Batches of oocytes were not used unless >95% had intact germinal vesicles prior to treatment and >95% underwent GVBD upon treatment with 6% 2.5 *M* KCl (Allen, 1953).

All antagonists and ionophore A23187 were obtained from Sigma Chemical Co. Chlorpromazine, verapamil, calmidazolium, N-(6-aminohexyl)-5-chloro-1-napthalenesulfonamide (W-7), and N-(6-aminohexyl)-1-napthalenesulfonamide (W-5) were made fresh before each experiment as a 10 m*M* solution in ethanol or dimethyl sulfoxide. Ionophore A23187 and cytochalasin B were stored as stock solutions at −20°C. Ionophore A23187 was made as a 2.5 m*M* stock in dimethyl sulfoxide (DMSO). Cytochalasin B was made as a 1 mg/ml stock in DMSO. Calmodulin antgonists were added to oocytes at the concentrations given in the figure legends. Ionophore A23187 was added to oocytes at a final concentration of 2.5 μM. This concentration is effective at initiating GVBD in this species (Schuetz, 1975; Eckberg, 1983). Cytochalasin B was added at various concentrations ranging from 0.03 to 3 μg/ml. Control experiments were always performed in which either DMSO or ethanol was added without antagonist.

Artificial seawater (ASW), calcium-free artificial seawater (CFSW), and calcium- and magnesium-free artificial seawater (CMFSW) were made according to standard MBL formulae (Cavanaugh, 1964). All artificial seawaters contained 10 m*M* TRIS-HCl, pH 8.2, to prevent possible pH-dependent nonspecific effects of the acidic antagonists (Allen, 1953; Carroll and Eckberg, 1983).

When GVBD was induced by KCl or ionophore, oocytes were usually exposed to the antagonist for 2 min prior to addition of KCl. In experiments in which GVBD was induced by fertilization, oocytes were inseminated with sufficient sperm to achieve synchronous fertilization, and antagonists were added 1 min later. Oocytes and embryos were examined by phase-contrast and Nomarski differential interference contrast microscopy at intervals for at least one hour after treatment for evidence of GVBD, ameboid contractions, and any other changes.

RESULTS

Calmodulin antagonists can block GVBD

Since KCl induces GVBD rapidly and synchronously in oocytes in this species, and KCl-induced GVBD would be independent of possible indirect inhibition through inhibition of fertilization, we initially tested the ability of calmodulin antagonists to prevent GVBD on KCl-activated oocytes. Figure 1 shows that all calmodulin antagonists tested blocked GVBD in *Spisula* oocytes when induced by KCl. The results also show that calmidazolium is maximally effective at *ca.* 3 μM, a substantially lower effective concentration than observed for chlorpromazine or W-7 (*ca.* 50–75 μM), and that W-5 is ineffective at concentrations up to 200 μM.

We also tested the effects of calmodulin antagonists on GVBD when induced by fertilization, the natural trigger in this species. Figure 2 shows that all calmodulin antagonists tested inhibited GVBD when induced by fertilization. Furthermore, the effective concentrations of the antagonists for fertilization-induced GVBD were similar to those for KCl-induced GVBD. Calmidazolium was maximally effective at *ca.* 3–10 μM, chlorpromazine and W-7 were maximally effective at *ca.* 75 μM, and W-5 was ineffective at any concentration up to 200 μM. The antagonists had no effect on post-GVBD development through first cleavage, because oocytes which successfully underwent GVBD in the presence of the inhibitors after fertilization also formed polar bodies and cleaved normally and synchronously with controls.

To determine the period of sensitivity to calmodulin antagonists for inhibition of GVBD, KCl-stimulated oocytes were exposed to 100 μM W-7 at various times after KCl stimulation and examined for GVBD 20 min later. Figure 3 shows that GVBD was prevented only if W-7 was added within 1 min after KCl addition. Similar results were obtained using calmidazolium (data not shown).

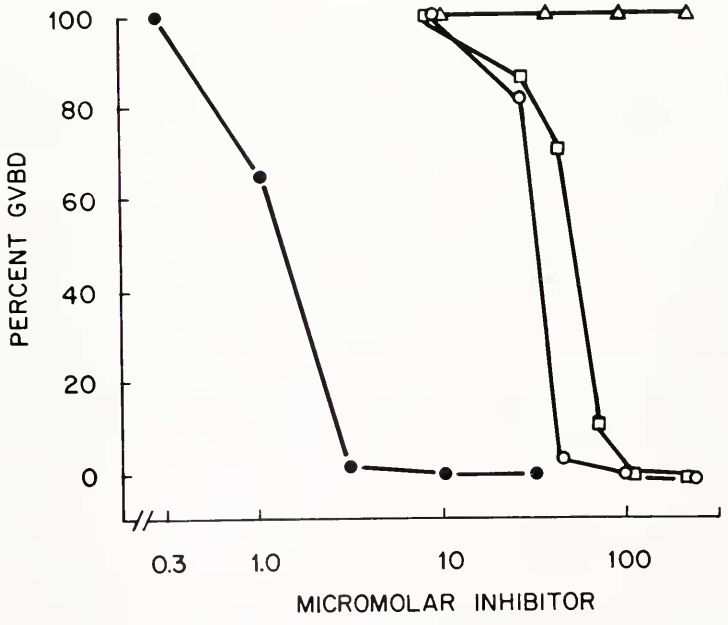

FIGURE 1. Effect of calmodulin antgonists on KCl-induced GVBD in *Spisula* oocytes. Oocytes were treated with different concentrations of calmidazolium (filled circles), chlorpromazine (open circles), W-7 (squares), or W-5 (triangles). Two minutes later they were exposed to 6% 2.5 *M* KCl and examined for GVBD 20 min later.

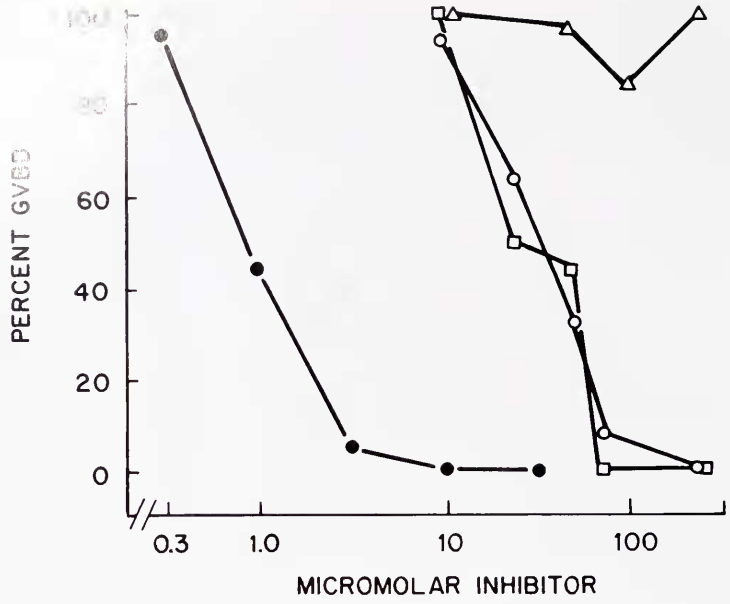

FIGURE 2. Effect of calmodulin antagonists on fertilization-induced GVBD in *Spisula* oocytes. Oocytes were inseminated, and 1 min later they were exposed to different concentrations of calmidazolium (filled circles), chlorpromazine (open circles), W-7 (squares), or W-5 (triangles). They were examined for GVBD 20 min later.

Calmodulin antagonists do not prevent ionophore activation of Spisula *oocytes*

Ionophore A23187 activation of *Spisula* oocytes apparently involves a different pathway than activation by KCl or fertilization (Eckberg, 1983). Specifically, ionophore activation should result directly from initiation of a calcium ion flux. Accordingly, we tested the effects of calmodulin inhibitors on ionophore A23187-induced GVBD. Table I shows that none of the drugs inhibited ionophore-induced GVBD.

A continued calcium flux is required after the calmodulin-dependent steps

The timing of the effect of calmodulin antagonists and the lack of inhibition of ionophore-induced GVBD by calmodulin antagonists suggested that a continued calcium flux after the calmodulin-dependent step might be required for GVBD in this species. Since calcium channel blockers are known to prevent the required calcium flux and GVBD (Guerrier *et al.*, 1981), we treated oocytes with the calcium channel blocker, verapamil. Figure 3 shows that verapamil blocks GVBD when added up to 2½ minutes after KCl treatment. Therefore, the calcium channels are required for 1–2 min after the calmodulin antagonist-sensitive period.

Oocytes treated with calmodulin antagonists undergo ameboid contractions without GVBD

Oocytes exposed to ≥ 50 μM chlorpromazine or W-7 or to ≥ 3 μM calmidazolium often underwent violent ameboid contractions. The contractions observed included formation and retraction of pseudopod-like protrusions and often resulted in net movement of the oocytes across the substratum. Initiation of ameboid contractions

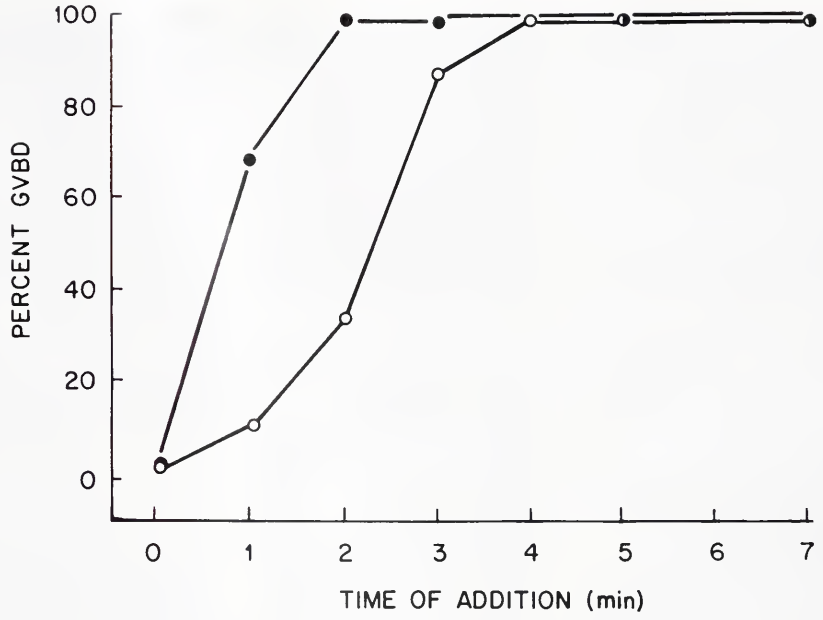

FIGURE 3. Time of sensitivity to W-7 and verapamil of KCl-induced GVBD in *Spisula* oocytes. Oocytes were exposed to 6% 2.5 *M* KCl at T = 0 min and 100 μM W-7 (filled circles) or 75 μM verapamil (open circles) was added at the times indicated. Oocytes were examined for GVBD 20 min after KCl addition. The times of addition at which GVBD was inhibited 50% were determined using curve-fitting software to be 0.94 ± 0.16 min for W-7 and 2.47 ± 0.69 min for verapamil. These times were significantly different from each other by the *t*-test ($P < 0.05$).

occurred in CFSW and CMFSW as well as in ASW and occurred whether or not oocytes had been previously inseminated or stimulated with KCl but only if the germinal vesicles had not broken down (data not shown). Examples of the kind of contractions and deformations observed are shown in Figure 4c, d, e. In many cases, these severe contractions obliterated the germinal vesicles (*cf.* Fig. 4e).

Observation of ameboid contractions suggested activation of a microfilament-dependent cytoplasmic contractile system. To test this, we simultaneously treated oocytes with 75 μM chlorpromazine and various concentrations of cytochalasin B (CB). As illustrated by Figure 4f, 0.3 μg/ml CB prevented the ameboid contractions. This treat-

TABLE I

Ineffectiveness of calmodulin antagonists at inhibition of GVBD induced by ionophore A23187

Antagonist[1]	% GVBD	±SD
none	98	2
calmidazolium	100	1
W-7	83	12
W-5	84	8
chlorpromazine	95	3
no ionophore	3	1

[1] Chlorpromazine, W-7, and W-5 were 100 μM; calmidazolium was 10 μM; ionophore A23187 was 2.5 μM. Three experiments were performed.

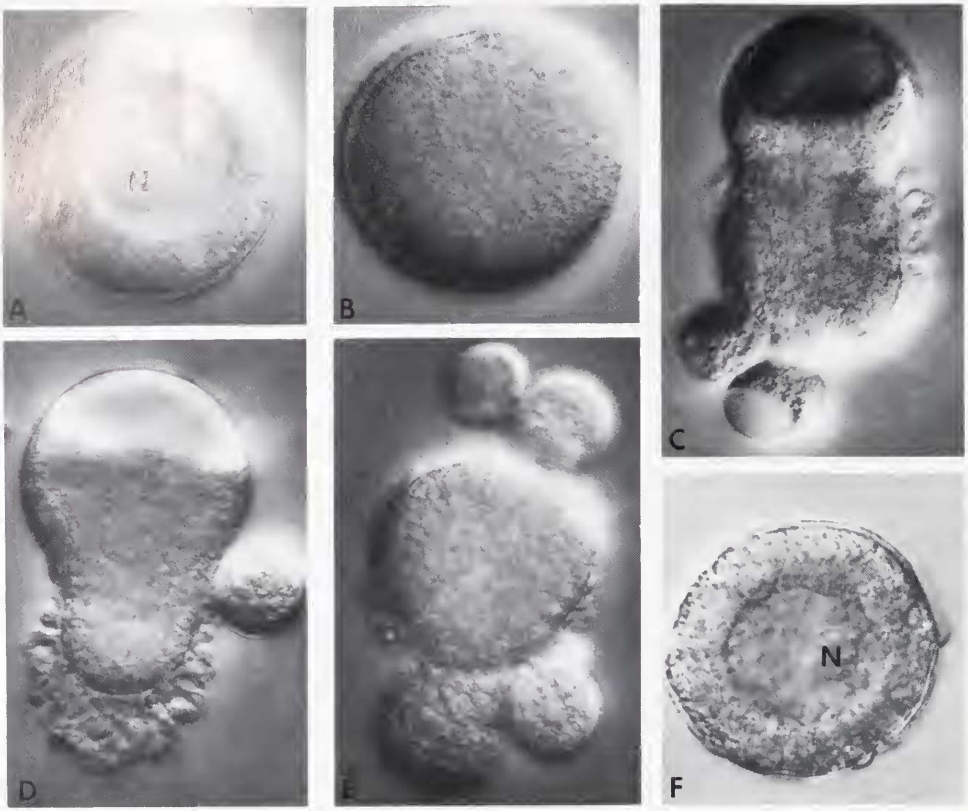

FIGURE 4. Ameboid contractions induced in *Spisula* oocytes by treatment with 75 μM chlorpromazine. A. Primary oocyte prior to treatment. B. KCl-stimulated oocyte that has undergone GVBD. C., D., E. Examples of oocytes showing severe ameboid contractions after treatment with 75 μM chlorpromazine. Note that in C the germinal vesicle is eccentric and contains a nucleolus, in D the eccentric remnant of the nucleolus lacks a nucleolus, and in E the germinal vesicle has been obliterated. This type of behavior is typical. F. An oocyte treated with 75 μM chlorpromazine and 0.3 μg/ml cytochalasin B. The ameboid contractions have been prevented. N = nucleolus.

ment blocked contractions when CB was added simultaneously with the calmodulin antagonist and stopped the contractions when added after they had begun. CB treatment also showed that the apparent obliteration of the germinal vesicles in ameboid oocytes (Fig. 4e) also resulted from the contractile activity. When the ameboid contractions were prevented, the germinal vesicles persisted (Fig. 4f).

DISCUSSION

The results of this study provide important new evidence concerning the involvement of calcium and calmodulin in germinal vesicle breakdown. Calmodulin involvement is demonstrated by the fact that several structurally different calmodulin antagonists block GVBD at concentrations similar to those at which they are known to affect calmodulin (Hidaka *et al.*, 1980; Weiss and Wallace, 1980), and that an analog of one of the antagonists which has little anticalmodulin activity has no effect on GVBD. Calmodulin had been implicated previously in GVBD in starfish (Meijer and Guerrier, 1981), amphibians (Wasserman and Smith, 1981), and the mouse (Bornslaeger *et al.*, 1984), which normally undergo GVBD in response to hormonal stimu-

lation and the annelid, *Chaetopterus,* (Carroll and Eckberg, 1983) which normally undergoes GVBD upon contact of the oocyte with seawater. *Spisula* normally undergoes GVBD in response to fertilization, and can also undergo GVBD in response to parthenogenetic stimulation (Allen, 1953; Schuetz, 1975). The results of this study demonstrate that, in this case as well, GVBD is a calmodulin-dependent event. Taken together with previous results, these studies strongly suggest that calmodulin involvement in GVBD is a general and therefore probably fundamental phenomenon.

The present results demonstrate that calmodulin is active at an early stage in GVBD, because addition of W-7 as little as 1 min after KCl activation was ineffective at inhibiting GVBD. Dorée *et al.* (1982) suggested that calmodulin acts at the oocyte surface and is involved in the transduction of the hormonal message in the initiation of GVBD in starfish because injected anticalmodulin drugs and antibodies were ineffective and inhibition of GVBD by external anticalmodulin drugs could be overcome by increasing the hormone concentration. The results of our experiments are consistent with a calmodulin effect at the cell surface because of the rapidity of GVBD inhibition by externally applied calmodulin antagonists. The results of the present study and our previous study on *Chaetopterus* extend the hypothesis of Dorée *et al.* to species in which GVBD is not hormone-dependent.

The results of the ionophore and verapamil studies provide additional information about the roles of calcium and calmodulin in the activation of the *Spisula* oocyte. None of the calmodulin antagonists blocked ionophore-induced GVBD, indicating that the calmodulin-dependent steps occur prior to the calcium flux. This also shows that the effects of the calmodulin antagonists in inhibiting GVBD do not result from generalized toxicity. Treatment with verapamil indicated that calcium channels must be open for 2–3 minutes for GVBD to occur. This period substantially exceeds the period of sensitivity to calmodulin antagonists. Together, these studies show that while calmodulin is required for GVBD, there is at least one additional calcium-sensitive event subsequent to the calmodulin-dependent step involved in the initiation of GVBD. These results imply that calmodulin is involved in the initiation of the required calcium uptake. Calmodulin has also been implicated in the regulation of calcium transport in erythrocytes (Muallem and Karlish, 1980; Larsen *et al.,* 1981).

Our experiments suggest the following model for activation. Sperm or a parthenogenetic agent contacts the surface of the oocyte, and initiates a reaction or series of reactions that involve calmodulin at an early step. These reactions, in turn, result in an increase in intracellular calcium, probably by uptake from the surrounding seawater (Guerrier *et al.,* 1981; Jaffe, 1985). This increase in intracellular calcium triggers the events that result in GVBD and polar body formation.

The activation of ameboid contractions by calmodulin antagonists had been reported previously in *Chaetopterus* oocytes (Carroll and Eckberg, 1983). In that species, ameboid contractions are a normal result of parthenogenetic activation (Lillie, 1902; Eckberg, 1981) and could therefore represent normal egg activation. In *Spisula,* however, ameboid contractions are not normally associated with egg activation, although several kinds of cytoplasmic movements have been reported to occur under certain conditions (Rebhun, 1963). The contractions reported here differ from those previously identified by Rebhun. The contractions observed in this study occurred in oocytes which had not undergone GVBD, whereas those in Rebhun's study took place only in eggs which had undergone GVBD.

While the violent ameboid contractions we observed sometimes resulted in cytolysis, they were not simply the result of cytolysis because when oocytes were treated with cytochalasin B simultaneously with the calmodulin antagonists, the contractions were blocked. Additionally, when CB was added to oocytes undergoing these contractions, they relaxed to a spherical shape. These contractions, therefore, represent a

cytoplasmic contractile activity that is microfilament-dependent and directly or indirectly initiated by calmodulin antagonists. Calmodulin antagonists have been shown previously to disrupt cytoskeletal organization in mammalian (Osborn and Weber, 1980) cells as well as sea urchin eggs (Carroll and Eckberg, unpub.) and embryos (Hagström and Lönning, 1973) and *Chaetopterus* oocytes (Carroll and Eckberg, 1983). The mechanism by which calmodulin antagonists initiate these contractions is unknown and currently under study.

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